# I. INTRODUCTION

## A. Purpose of the Study

The purpose of this study is to perform an Independent Laboratory Validation (ILV) of BASF Analytical Method D0301: "THE DETERMINATION OF RESIDUES OF BAS 320 I AND ITS METABOLITES, M320I04 (REG. NO.4096485), M320I06 (REG. NO. 121464), AND M320I23 (REG. NO. 4984051) IN SOIL USING LC-MS/MS" and to demonstrate that the Method D0301 can be performed with acceptable recoveries at an outside facility.

## B. Summary of the Results

The independent laboratory validation of the BASF method was successfully completed. The first trial met the criteria for a successful validation of the method for soil for BAS 320 I and its metabolites. Communication between the performing laboratory and the study monitor was not needed for successful completion of the method validation.

# II. SAMPLE HISTORY

Homogenized control soil, BASF sample code RSN 2002502 – 0251 (Study No. 67702) was provided by BASF. Soils with RSN 2002502 are from Texas and have been identified as Sandy Clay loam and with high water content (>12 % moisture) at 36-42 inches. This soil was chosen for this study so that the matrix will be harder to work with in comparison to other soils. The aliquots taken for control and fortification purposes were given a unique lab code number (i.e. 2K3-903-83437-01). The sample was received on 8/21/03.

# III. PROCEDURE - METHOD SYNOPSIS

BASF Draft Analytical Method D0301, dated March 11, 2003, was used to determine residues of BAS 320 I and its metabolites, M320I04, M320I06, and M320I23. Following is a summary of the method:

A 10 g soil sample aliquot is extracted by shaking with methanol, followed by methanol-water, 50:50, v/v. The volume of the combined extract is adjusted to 100 mL. An aliquot of the extract (10%) is evaporated to 2-3 mL. The sample is then diluted with methanol to volume for HPLC-MS/MS determination. The method has a limit of quantitation of 0.01 mg/kg (0.01 ppm) in soil for each analyte.

# IV. LIMIT OF QUANTITATION AND DETECTION

The limit of quantitation (LOQ) was set in the method to 0.01 ppm and can be defined as the lowest fortification level tested for all compounds. With the instrument parameters optimized at ADPEN, the response for the compounds at the LOQ was at least 10 times better than the noise level. The limit of detection was not determined by the method, but set at 20 % of the limit of quantitation. The lowest standard for each analyte in the calibration curve had good detectability with a signal to noise ratio greater than 3:1. The instrument used was a PESciex API 365, which is less sensitive than the API 4000 used in the method. The injection size was increased from 10 µL to 50 µL. The 0.01-ppm fortification level is an acceptable limit of quantitation even for instruments less sensitive than the API 4000.

## V. CALIBRATION, CALCULATIONS AND STATISTICS

Residues of BAS 320 I (E and Z- isomers) and its metabolites, M320I04, M320I06 and M320I23 were quantitated by external standard. A calibration curve for each analyte was generated by plotting the detector's response in peak area versus pg of standard injected. The data system derived an equation for the fit of the standard curve and this equation was used to calculate intercept and slope of the linear regression curve.

Peak integration and quantitation were performed using computer and integration software. PE Sciex Analyst® data system was used for LC/MS/MS. PPM calculations and Recovery results were computed for each set of samples by Microsoft's Excel® and reported in a spreadsheet data report. Equations used for quantitation are presented in Figure 2. Statistical treatment of the data included calculation of averages and standard deviations and is presented in Tables 1 and 2. These calculations were performed using Excel. Results were rounded off for reporting purposes but not for any calculations. The calibration curve was obtained by direct injection of 50 µL of the mixed BAS 320 I (E and Z- isomers) and its metabolites, M320I04, M320I06 and M320I23 standards for LC-MS/MS in the range of 1.0 pg/µL to 20.0 pg/µL. In a given injection run, the same injection volume is used for all samples and standards. Typical standard amounts injected on-column range from 50 to 1000 pg.

The transitions monitored were 505.1 → 301.9 for BAS 320 I (E and Z- isomers), and 288.1→ 141.8, 146.0 → 101.9, and 519.1 → 185.0 for M320I04, M320I06 and M320I23, respectively.

**TABLE 3. Typical Instrument Parameters for Analysis of BAS 320 I and its Metabolites**

Instrument

| | |
|---|---|
| High Performance Liquid Chromatograph (HPLC): | Hewlett Packard 1100 Series, Instrument No. 8 |
| LC-MS/MS: | PESciex API 365 LC-MS/MS with Turbo IonSpray Inlet |
| Data Acquisition System: | Analyst 1.3 |

HPLC Parameters

| | |
|---|---|
| Column: | Inertsil ODS-3, 150mm x 2.0 mm, 5µ |
| Mobile Phase: | A: Water + 4mM ammonium acetate and 0.1% acetic acid<br>B: Methanol with 4mM ammonium acetate and 0.1% acetic acid |
| Injection Volume: | 50 µL |
| Column Temperature: | 30°C |

Pump Timetable:

| Time (min.) | %A | %B | Flow Rate (µL/min.) |
|---|---|---|---|
| 0.0 | 70.0 | 30.0 | 400 |
| 2.0 | 15.0 | 85.0 | 400 |
| 5.0 | 15.0 | 85.0 | 400 |
| 6.0 | 0.0 | 100.0 | 400 |
| 7.50 | 0.0 | 100.0 | 400 |
| 8.0 | 70.0 | 30.0 | 400 |
| 11.0 | 70.0 | 30.0 | 400 |

## TABLE 3. Typical Instrument Parameters for Analysis of BAS 320 I and its Metabolites (continued)

LC/MS/MS Parameters

Scan Type: MRM (MRM)
Polarity: Negative
Scan Mode: N/A
Ion Source: Turbo Spray
Resolution Q1: UNIT
Resolution Q3: UNIT
Intensity Thres.: 0.00 cps
Smart Settling: On
Settling Time: 0.0000 msec
MR Pause: 5.0000 msec
MCA: No
Step Size: 0.00 amu

| Q1 Mass (amu) | Q3 Mass (amu) | Dwell (msec) | Param | Start | Stop |
|---|---|---|---|---|---|
| 505.19 | 302.05 | 150.00 | DP | -106.00 | -106.00 |
| | | | FP | -330.00 | -330.00 |
| | | | EP | -7.50 | -7.50 |
| | | | CEP | -14.00 | -14.00 |
| | | | CE | -24.00 | -24.00 |
| | | | CXP | -18.00 | -18.00 |

| Q1 Mass (amu) | Q3 Mass (amu) | Dwell (msec) | Param | Start | Stop |
|---|---|---|---|---|---|
| 505.19 | 301.85 | 150.00 | DP | -106.00 | -106.00 |
| | | | FP | -320.00 | -320.00 |
| | | | EP | -9.00 | -9.00 |
| | | | CEP | -90.12 | -90.12 |
| | | | CE | -24.00 | -24.00 |
| | | | CXP | -16.00 | -16.00 |

| Q1 Mass (amu) | Q3 Mass (amu) | Dwell (msec) | Param | Start | Stop |
|---|---|---|---|---|---|
| 288.06 | 142.05 | 150.00 | DP | -56.00 | -56.00 |
| | | | FP | -240.00 | -240.00 |
| | | | EP | -4.00 | -4.00 |
| | | | CEP | -96.62 | -96.62 |
| | | | CE | -38.00 | -38.00 |
| | | | CXP | -10.00 | -10.00 |

| Q1 Mass (amu) | Q3 Mass (amu) | Dwell (msec) | Param | Start | Stop |
|---|---|---|---|---|---|
| 145.98 | 101.85 | 150.00 | DP | -81.00 | -81.00 |
| | | | FP | -300.00 | -300.00 |
| | | | EP | -6.50 | -6.50 |
| | | | CEP | -100.87 | -100.87 |
| | | | CE | -14.00 | -14.00 |
| | | | CXP | -10.00 | -10.00 |

| Q1 Mass (amu) | Q3 Mass (amu) | Dwell (msec) | Param | Start | Stop |
|---|---|---|---|---|---|
| 519.11 | 184.95 | 150.00 | DP | -51.00 | -51.00 |
| | | | FP | -270.00 | -270.00 |
| | | | EP | -1.00 | -1.00 |
| | | | CEP | -89.70 | -89.70 |
| | | | CE | -54.00 | -54.00 |
| | | | CXP | -12.00 | -12.00 |

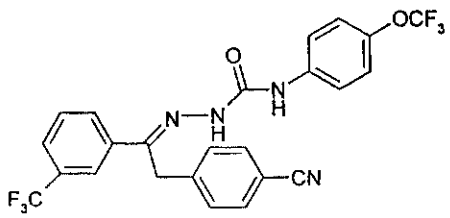
| Q1 Mass (amu) | Q3 Mass (amu) | Dwell (msec) | Param | Start | Stop |
|---|---|---|---|---|---|
| 519.11 | 287.65 | 150.00 | DP | -51.00 | -51.00 |
| | | | FP | -270.00 | -270.00 |
| | | | EP | -1.00 | -1.00 |
| | | | CEP | -89.70 | -89.70 |
| | | | CE | -28.00 | -28.00 |
| | | | CXP | -14.00 | -14.00 |

Parameter Table (Period 1 Experiment 1):
NEB: 8.00
CUR: 8.00
IS: -4200.00
TEM: 425.00
GAD: 2.00

**FIGURE 1. Structure of the Test and Reference Substance**

Fortification and LC-MS standards

| | |
|---|---|
| BASF Code Name: | BAS 320 I (E-Isomer) |
| BASF Registry Number: | 4102472 (CL 814027) |
| CAS Number: | 139968-49-3 |
| Chemical Name: | 2-[2-(4-Cyanophenyl)-1-[3-trifluoromethyl)-phenyl]-ethylidene]-N-[4-(trifluoromethoxy)-phenyl]-hydrazinecarboxamide (E) |
| Molecular Formula: | $C_{24}H_{16}F_6N_4O_2$ |
| Molecular Weight: | 506.4 |
| Appearance: | White powder |
| Water Solubility: | 0.57 µg/L |
| Lot Number: | AC12145-19C |
| Purity: | 98.7% |
| Structural Formula: | |




| | |
|---|---|
| BASF Code Name: | BAS 320 I (Z-Isomer) |
| BASF Registry Number: | 4102572 (CL399260) |
| CAS Number: | 139970-56-2 |
| Chemical Name: | 2-[2-(4-Cyanophenyl)-1-[3-trifluoromethyl)-phenyl]-ethylidene]-N-[4-(trifluoromethoxy)-phenyl]-hydrazinecarboxamide (Z) |
| Molecular Formula: | $C_{24}H_{16}F_6N_4O_2$ |
| Molecular Weight: | 506.4 |
| Appearance: | White powder |
| Water Solubility: | 1.22 µg/L |
| Lot Number: | AC12705-150-P2 |
| Purity: | 96.9% |
| Structural Formula: | |

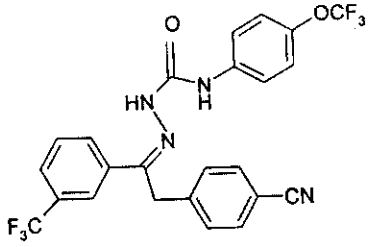
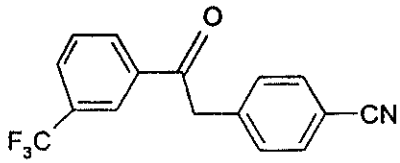


**FIGURE 1. Structure of the Test and Reference Substance (continued)**

| | |
|---|---|
| BASF Code Name: | M320I04 |
| BASF Registry Number: | 4096485 (CL 397864) |
| CAS Number: | 146653-56-7 |
| Chemical Name: | p-[m-(Trifluoromethyl)-phenacyl]benzonitrile |
| Molecular Formula: | $C_{16}H_{10}F_3NO$ |
| Molecular Weight: | 289.3 |
| Appearance: | Reddish brown powder |
| Lot Number: | AC12705-149-P |
| Purity: | 97.5% |
| Structural Formula: | |




| | |
|---|---|
| BASF Code Name: | M320I06 |
| BASF Registry Number: | 121464 (CL 25945) |
| CAS Number: | 619-65-8 |
| Chemical Name: | p-Cyanobenzoic acid |
| Molecular Formula: | $C_8H_5NO_2$ |
| Molecular Weight: | 147.13 |
| Appearance: | White powder |
| Lot Number: | AC12859-10 |
| Purity: | 93%; re-assayed 12/19/2003: 87.3% |
| Structural Formula: | |

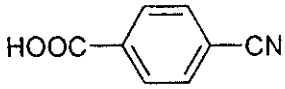
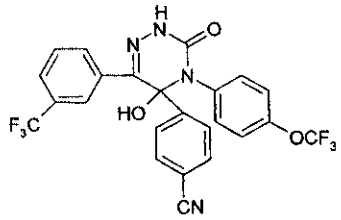


**FIGURE 1. Structure of the Test and Reference Substance (continued)**

| | |
|---|---|
| BASF Code Name: | M320I23 |
| BASF Registry Number: | 4984051 |
| Chemical Name: | 4-[5-hydroxy-3-oxo-4-{4-(trifluoromethoxy)phenyl}-6-{3-(trifluoromethyl)phenyl}-2,3,4,5-tetrahydro-1,2,4-triazin-5-yl]benzonitrile |
| Molecular Formula: | $C_{24}H_{14}F_6N_4O_3$ |
| Molecular Weight: | 520.39 |
| Appearance: | White powder |
| Lot Number: | 2059004 |
| Purity: | 98.8% |
| Structural Formula: | |



## FIGURE 2. Typical Calculations for the Quantitation of Residues in Soil

Recovery results are calculated by comparison to the standard calibration curves obtained from a linear regression analysis of the data found by the data system. The equation for the fit of the standard curve was used to calculate intercept and slope of the linear regression curve. The intercept and the slope were used in the equation for quantitation. Excel is used to calculate the ppm and percent recovery and to present the data in a report format. The following equations were taken from the method and were used for quantitation:

The recoveries and residues of BAS 320 I and its metabolites in ppm are calculated with the following formulas:

**Residue in ppm** (ppm Found) = $\frac{\text{pg found per injection}}{\text{mg injected}} \times \frac{\text{ng}}{1000\ \text{pg}}$

**Percent recovery (%)** = $\frac{\text{Residue (ppm) for [fortified sample - control sample]}}{\text{Amount (ppm) fortified}} \times 100$

**pg found per injection** = Amount of Analyte calculated from calibration curve

**Standard curve: pg** = $\frac{\text{Peak Area – intercept}}{\text{Slope}}$

**mg injected** = $\frac{\text{Sample weight (10 g) extracted}}{\text{Final extraction volume (100 mL) [Section 3.2.4]}} \times (\mu\text{L injected}) \times (\text{aliquot factor (F1 x F2)})$

**First Aliquot factor (F1)** = $\frac{\text{Aliquot taken [Section 3.2.5]}}{\text{Final volume [Section 3.3.1]}} = \frac{10\ \text{mL}}{5\ \text{mL}} = 2.0$

(Samples with residue at the limit of quantitation, 0.01 ppm)

**Second Aliquot factor (F2) =** 1, 0.1 and 0.01 for 0.01, 0.1 and 1.0 ppm fortification samples, respectively.

**FIGURE 2. Typical Calculations for the Quantitation of Residues in Soil**

As an example, calculations to obtain BAS 320 I (E isomer) percent recovery values using sample 2K3-903-83437-04 (Recovery) are shown below.

$$\textbf{Residue in ppm} = \frac{85.5\ \text{pg}}{10.0\ \text{mg}} \times \frac{1\ \text{ng}}{1000\ \text{pg}}$$

$$= 0.00855$$

$$\textbf{Percent recovery (\%)} = \frac{0.00855\ \text{ppm} - 0.00000\ \text{ppm}}{0.01\ \text{ppm}} \times 100$$

$$= 85.5\%$$

**ng found per injection =** 0.0855 or 85.5 pg

$$\textbf{Standard curve: pg} = \frac{3533 - (-137)}{42.9}$$

$$= 85.5$$

$$\textbf{mg injected} = \frac{10\ \text{g}}{100\ \text{mL}} \times 50\mu\text{L} \times (2.0 \times 1)$$

$$= 10.0\ \text{mg}$$

$$\textbf{First Aliquot factor (F1)} = \frac{10\ \text{mL}}{5\ \text{mL}}$$

$$= 2$$

**Second Aliquot factor (F2) =** 1